2. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

2.1 Introduction

High Performance/Pressure Liquid Chromatography (HPLC) is a liquid chromatographic technique in which the separation of the components from a mixture is achieved by pumping mobile phase at high pressure through a suitably modified column. Since the mobile phase is delivered under high pressure by using appropriate displacement pumps or gas pressure, the technique has derived its name as high pressure. Moreover, high performance in the name indicates that HPLC offers improved performance than other conventional column chromatographic techniques. This is because it utilizes a stationary phase which has very small particle size (3-20 µm) and provides a greater surface area. This in turn increases the elution rate by over 100 folds, thereby rendering the method best suited for efficient separation of complex mixtures. In simple terms, HPLC technique is an improved version of conventional column chromatography. Moreover, now-a-days all the liquid chromatographies (LC) are done by employing pressurized flow. Therefore, the terms LC and HPLC are interchangeable.

Advantages

1. It offers high separation efficiency and speed of analysis.
2. It offers high degree of selectivity for specific analysis of components due to availability of wide variety of solvents and column packings.
3. It requires less amount of sample and allows continuous monitoring of the column effluent.
4. Since it can operate with wide choice of mobile and stationary phases, it can be used in the analysis of polar and ionic substances, metabolic products, thermolabile as well as nonvolatile substances.
5. The results obtained are precise, accurate and reproducible.
6. It allows complete separation of components from the mixture for further analysis and characterization.
7. Adsorption, partition, ion exchange and exclusion column separation can be done by HPLC.
8. Modern HPLC instruments can be easily integrated with computer systems which makes the process easy and also helps in storing the results.
9. Various developmental techniques of the chromatogram like isocratic elution, gradient elution etc., are possible with HPLC.

Disadvantages

1. Maintenance cost of the equipment is high.
2. Preparation of the sample is laborious and time consuming.
3. HPLC columns are expensive.
4. Pretreatment procedures like degassing and filtration are required for solvents.
5. Skilled and well trained personnels are required for carrying out the procedure.
6. Large amounts of solvent (mobile phase) is required for separation.
7. *In situ* derivatization of the components is not possible.
8. Being a closed system, visual identification of the components is not possible.

2.2 Principle

The basic principle involved in HPLC is adsorption. In this, a small volume of liquid sample is injected into the column packed with a suitable stationary phase. The sample is then forced through the column by delivering the mobile phase at very high pressure. The mixture of the components gets separated based on their relative affinities for the stationary phase. The components which have the least affinity for stationary phase get eluted faster than the components with higher affinities.

2.3 Theory

The theory of HPLC and GC is similar and for detailed discussion refer theory of GC. Only the differing aspects in the theory of HPLC are described below.

Retention Volume (V_R)

The volume of the mobile phase required for elution of 50% of a component from the column is called retention volume.

$$V_R = R_t \times \text{flow rate}$$

Asymmetry Factor

Theoretically, the peaks obtained in chromatograms should be symmetrical about their centre and follow Gaussian distribution. However, symmetrical peaks are not obtained due to fronting or tailing.

Fronting

Fronting is the term used when the left side of the peak is broader than the right side. Asymmetry factor in case of fronting is less than one.

Reason: The sample applied may be greater in quantity, which results in saturation of the stationary phase. It should be reduced to get a more uniform peak.

Tailing

Tailing of a peak occurs when the right side of the peak is broader compared to the left side. Asymmetry factor in case of tailing is greater than one.

Reason: Tailing of peak occurs due to greater adsorption of sample onto the stationary phase. This is because of greater number of active adsorption sites on the solid support. Tailing can be minimized by treating the support suitably before use and by the use of a more polar mobile phase.

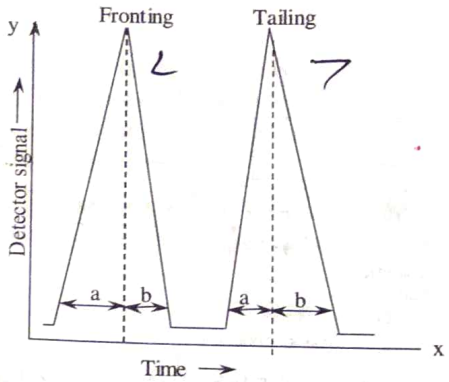

Figure: Fronting and Tailing

Calculation of Asymmetry Factor

Asymmetry factor of the peaks can be calculated using the formula,

$$AF = \frac{b}{a}$$

Where,

AF = Asymmetry factor

a and b = Distances of sides of the peak from the line of symmetry.

a and b are determined by taking points on the sides such that they lie at 5% or 10% of the peak height. Asymmetry factor should be in between 0.95 - 1.05. Its value is 1 for an ideal symmetric peak.

2.4 Instrumentation

A typical HPLC instrument consists of the following,

1. Mobile phase reservoirs and solvent pretreatment systems
2. Solvent delivery pumps
3. Precolumn/Guard column
4. Sample injection systems
5. Analytical columns
6. Detectors
7. Recorders and integrators.

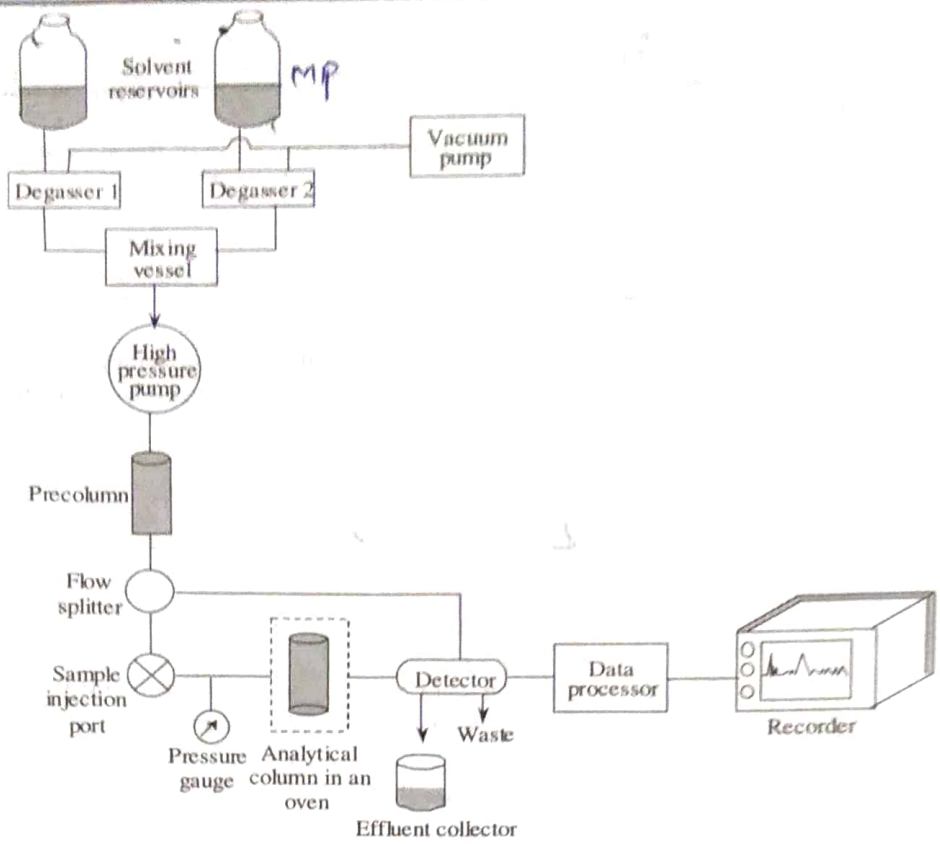

Figure: Schematic Representation of High Performance Liquid Chromatography

1. Mobile Phase Reservoirs and Solvent Pretreatment Systems

Mobile phase selected in HPLC may be either a pure solvent or a mixture of two or more solvents having appropriate eluting power for the sample components. The selection is mainly based on the polarity of the stationary phase, the nature of the sample components and upon the chromatographic method. Other properties like boiling point, flammability, viscosity, toxicity, pH and detector compatibility of the solvents are also considered. For isocratic elution, either one or a mixture of two or more solvents with fixed composition is used, whereas for gradient elution, the composition of the solvents used is changed continuously.

Glass or stainless steel reservoirs (of capacity 500 ml or more) are used in modern HPLC instruments for storing the mobile phase. The material chosen for construction of the mobile phase reservoirs should not be reactive.

Mobile phase used should be a pure solvent devoid of any particulate matter or gases. Degassers and filteration systems are often provided with the reservoirs.

2. Solvent Delivery Pumps

In HPLC, the mobile phase is passed through the stationary column under high pressure, by means of pumps. The pumps used in HPLC should possess the following features.

1. They should generate high pressures upto 6000 psi.
2. They should provide a flow rate ranging from 0.1 to 10 ml/min, flow control and flow reproducibility upto 0.5%.
3. All the components of the pump should be resistant to the solvents employed and must give a pulse free output.
4. They should be easy to dismantle and repair.
5. They should be adaptable for replacing solvents (or changing their proportions) in case of gradient elution HPLC.

The following pumps are used in HPLC.

Displacement Pumps

They act by maintaining a constant flow of the mobile phase throughout the column and are not affected by any changes occurring in the column. In these pumps, the constant solvent flow in the column is maintained by a plunger in the syringe-like chamber. The plunger is driven by a motor.

Advantages

1. Pulse free output is obtained.
2. The flow obtained does not depend either on viscosity of the solvent or on column back-pressure.

Disadvantages

1. Limited solvent capacity of about 200-500 ml.
2. Periodic change of solvents, in case of gradient elution is difficult.

Reciprocating Pumps

They consist of a small chamber in which the mobile phase is pumped by the forward and backward movement of a piston which runs on an electric motor. The flow of the mobile phase in the chamber is regulated by two valves. The piston directly pushes the mobile phase as both are in direct contact with each other or it pushes the mobile phase via a diaphragm which lies between it and the mobile phase.

The reciprocating pumps usually require damping devices which minimize the pulsed flow. Examples of damping devices include triple-headed pump, tube with flexible bellows, restrictors, etc. Piston pumps are a type of reciprocating pumps which are widely used in HPLC.

Advantages

1. They have a small internal volume (35 to 400 µl), but give an output pressure as high as 10,000 psi.
2. Gradient elution can be carried out without much difficulty.
3. The flow rate obtained is constant and independent of solvent viscosity and column back-pressure.
4. They possess a large solvent capacity.

Single Acting Piston Pump

It was the first reciprocating pump to be used for HPLC and is still the most popular pump for liquid chromatography.

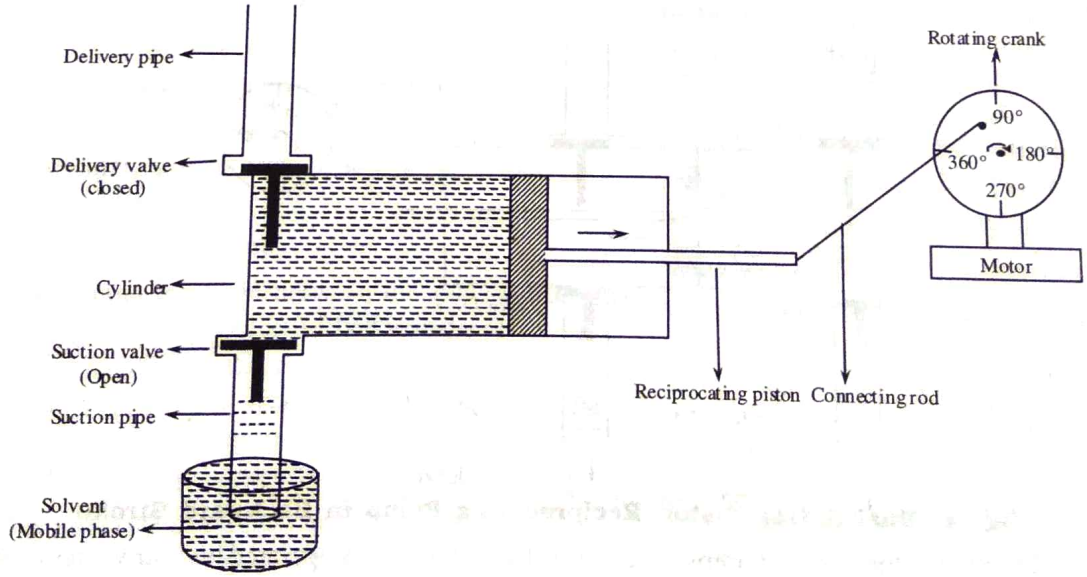

Figure: Single Acting Piston Reciprocating Pump in Backward Stroke

Working

Single acting piston reciprocating pump consists of a stationary cylinder with a piston inside it that moves to and fro. Two pipes i.e., the suction pipe and the delivery pipe are connected to this cylinder via two non-return unidirectional valves, the suction valve and the delivery valve respectively. The cylinder receives mobile phase via the suction valve from the suction pipe, while it delivers the mobile phase to the column from the delivery valve via delivery pipe.

The piston is connected to a crank by means of a connecting rod. The crank in turn is connected to an electric motor. When the piston is towards the extreme left of the cylinder, the cylinder is completely empty. Upon rotating the crank through 180°, the piston moves towards its right. This backward stroke creates partial vacuum in the cylinder. As a result, the suction valve is forcefully opened and pushed into the cylinder. When the cylinder completely fills up with the mobile phase, the suction valve automatically closes. At this point the piston is towards the extreme right of the cylinder, crank is at 180° and the suction valve is completely closed.

Now when the crank is rotated from 180° to 360°, the piston moves towards the extreme left. During this downstroke, the pressure of the mobile phase rises above the atmospheric pressure, thereby causing the delivery valve to open. The mobile phase is then delivered to the column through the delivery pipe via delivery valve. At this point the piston is towards the extreme left of the cylinder, crank is at 360° (completion of one revolution) and both the valves are closed and the cycle continues.

Advantages

1. Simple in design and relatively inexpensive.
2. Highly viscous liquids can be pumped.
3. These pumps are readily adaptable to gradient elution.
4. Solvent reservoir is unlimited.
5. Flow rate of mobile phase can be easily changed by adjusting the rpm of the crank.

Disadvantages

1. Maintenance cost is high.
2. Produces pulsed flow that causes detection problems and prevents good quantitative analysis.
3. Causes early column failure.

Dual Acting Piston Pump

These pumps are more efficient than single acting piston pumps as they provide almost pulse-free flow of mobile phase.

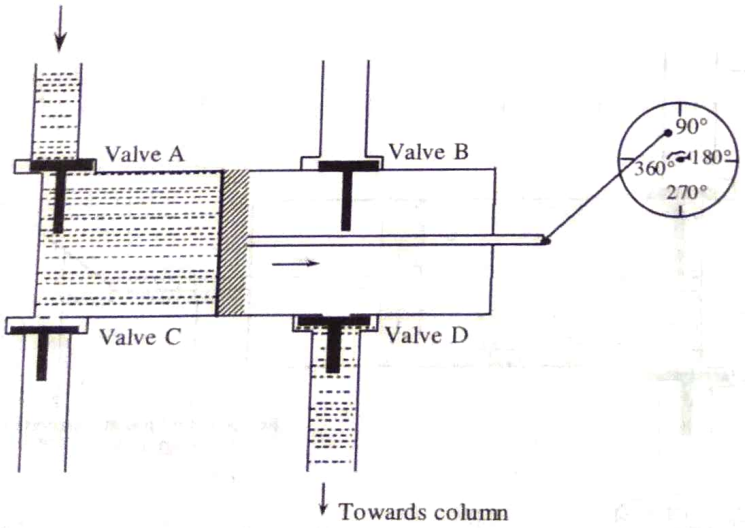

Figure: Dual Acting Piston Reciprocating Pump in Backward Stroke

Unlike single acting pumps, these pumps are equipped with four pipes operated by four valves of which two are suction valves and the other two are delivery valves. The fluid enters and exits the cylinder via two valves simultaneously.

In the above figure, when piston is in the backward stroke, mobile phase enters the cylinder via valve A and exits through valve D towards the column. Later when the piston is in the downward stroke, the mobile phase enters the cylinder through the valve B and exits through valve C. The whole process is repeated as with single acting piston pumps.

Advantages

1. Internal volume can be made very small (35-400 µl).
2. High output pressure upto 10,000 psi can be obtained.
3. Gradient elution of solvents is possible.
4. Constant flow rate which is independent of column back-pressure and solvent viscosity.
5. Provides almost pulse-free flow.

Constant Pressure Pumps

These pumps give a non-pulsating solvent flow but have very small capacity. Pneumatic pumps also known as non-reciprocating pumps are an example of constant pressure pumps. In these pumps, the mobile phase is placed in a collapsible container and highly pressurized gas is driven into the pump. This gas in turn displaces the solvent thereby enabling its transfer from the pump into the column. However, proper care should be practised to prevent the interaction of the pressurized gas with the eluting solvent as this may give rise to improper results.

Advantages

1. Pulse-free output.
2. Inexpensive compared to other pumps.

Disadvantages

1. Offers limited capacity and pressure output of 2000 psi.
2. Flow rate and the pressure output depend on solvent viscosity and column back-pressure.

3. Precolumn/Guard Column

A guard column is placed between the analytical column and sample injector port and is filled with the same packing material as that of the analytical column. It protects the analytical column from damage or loss of efficiency by strongly adsorbing the particulate matter or impurities present in the sample or the solvent.

These are made up of stainless steel that helps them withstand high pressures of about 5000 psi. They are usually short in length ranging between 2-20 cm. Smaller inner diameter (2-8 mm) of precolumns give good results and helps to increase their working efficiency.

They are generally packed with an inactive material of larger particle size, impregnated with 20-30% of a suitable silicone phase. Due to the larger particle size, the pressure drop across the precolumn is negligible when compared with that of the analytical column.

Significance

1. Precolumns enhance the life-span of the analytical columns by protecting them from dust, dirt, undissolved salts and particles present in the mobile phase or sample.
2. They are easy to replace or repack upon their contamination.
3. They help to filter the solid particles flowing from the pump.
4. Samples like polymers or salts that contain non-vaporizable residues can be easily analyzed without affecting the packing of the analytical column.
5. Helps to remove the sample components that bind irreversibly with the stationary phase.

4. Sample Injection Systems

In simple HPLC systems, the sample is introduced with an injection valve whereas in sophisticated HPLC systems, an autosampler provided with a microprocessor is used. The sample is delivered to the column head ensuring that the column packing material remains undisturbed. Three types of sample injectors are mostly used in HPLC.

(a) Syringe Injectors/Septum Injectors

In these syringe injectors, self-sealing elastomeric septum is used through which the sample is introduced into a thin layer of glass beads or teflon placed above the column. The material used for constructing these injectors should be able to tolerate pressures as high as 1500 psi. It gives efficient chromatographic separation.

(b) Stop Flow Injectors

In these injectors, an injection valve is used to control the solvent flow which stops the flow of mobile phase for a moment. The fitting at the column head is removed and sample is introduced at the top of the packing under normal atmospheric pressure. After sample application, the fitting is replaced and the system is again maintained at high pressure.

(c) Loop Injectors/Rheodyne Injectors

They are currently being used in automated systems for injecting small amount of sample (upto 10 µL).

It utilizes a sample loop which can be operated in two modes i.e., sampling mode and injection mode. In the sampling mode, the sample is introduced into the loop at normal atmospheric conditions. In the injection mode, the sample is injected into the column.

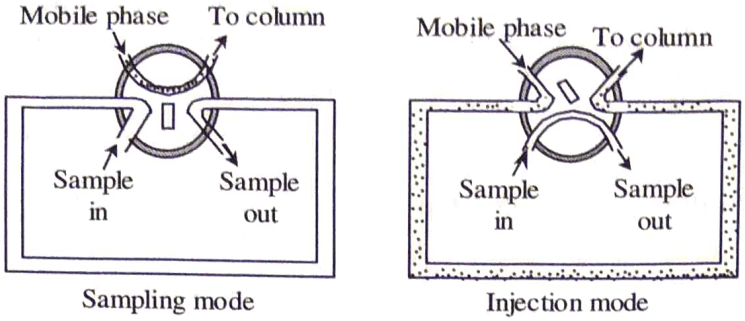

Figure: Operation of a Sample Loop

5. Analytical Columns

Analytical column is the central component of HPLC instrument where the actual separation of the components occur. Therefore, proper selection of the chemical nature and dimensions of the column is necessary as it affects the resolution, peak symmetry and the speed of analysis.

Columns made up of either stainless steel, heavy glass, polyethylene or polyethylene ether ketone (PEEK) are used. Stainless steel columns are preferred as they can withstand high pressures. When columns made of heavy glass are used, the system is maintained at pressures lower than 600 psi.

The dimensions of the column are selected based on the type of separation to be achieved. For carrying out analytical separation, columns of length 25 - 100 cm and internal diameter of 2-6 mm is selected whereas for preparative separation, columns of length 25 - 100 cm and internal diameter of 6 mm or more is utilized.

Types of Column

Two types of columns mainly used are conventional columns and microbore columns. Both these columns are similar in their construction, packing material and mobile phase selection but vary in the operating pressure and in performance. Microbore columns offer better performance compared to the conventional columns.

Table: Types of Analytical Columns

	Features	Conventional columns	Microbore column
1.	Column		
	❖ Material	Stainless steel	Stainless steel
	❖ Dimensions	Length : 3, 10, 15, 20, 25 cm Internal diameter : 4.6 mm	Length : 25 to 50 cm Internal diameter : 1 to 2 mm
2.	Stationary phase (packing material)	Porous, microparticulate silica, chemically modified silica or styrene/divinyl benzene copolymers	Same as conventional column
3.	Operating pressure	500-3000 psi	1000-5000 psi
4.	Mobile phase	❖ *For Normal Mode* : Hydrocarbons and chlorinated solvents or alcohols ❖ *For Reverse Mode* : Methanol or acetonitrile with water or aqueous buffer	❖ *For Normal Mode* : Same as conventional columns ❖ *For Reverse Mode* : Same as conventional columns
5.	Solvent flow rate	1-3 cm^3/min	10-100 µL/min
6.	Performance	Less efficient	More efficient

Column Packing Material (Stationary Phase)

1. *Microporous Supports*

These are totally porous particles, composed of silica, alumina or ion-exchange resins with a diameter of 5-10 μm. Their pore diameter is inversely related to the specific surface area. Since their particle size is very less, microporous supports offer large surface area (50-500 m^2/g). Moreover, these particles get easily packed into the HPLC columns.

2. *Pellicular Supports/Superficially Porous Supports/ Controlled Surface Porosity Supports*

They comprise of a central solid and rigid core (40 μm in diameter) covered by a layer (1-3 μm) of porous particles. The solid spherical core are generally glass beads which are coated with either silica, alumina or other adsorbent. Although, these supports are highly efficient, they offer a surface area of only 5-15 m^2/g. Thin porous layers of adsorbents over the glass beads enhance the rate of mass transfer whereas thick coatings although offer increased sample capacity, are associated with slow mass transfer.

E.gs: Corasil, Pellosil, Perisorb, Vydac, Zipax.

6. Detectors

The main function of detector in HPLC is to monitor the column effluent and also to detect the amount of sample present in it. The detectors emit electrical signals that are directly proportional to a certain property or characteristic of either the sample or the mobile phase. The detectors used in HPLC should possess the following features.

(a) They should possess high sensitivity and should be able to detect very low levels of solute.

(b) They should be cheap, reliable, easy to use, stable and non destructive.

(c) They should detect all the components in the sample in a wide range of mobile phases.

(d) They must be unaltered by changes in the temperature and flow rate.

(e) They should not lead to zone spreading.

The detectors used in HPLC are classified as follows.

(a) *Bulk property detectors* measure the property of the mobile phase both in presence and absence of sample.

E.gs: Refractive index detectors, visible detectors, conductivity detectors.

(b) *Solute property detectors* measure the property possessed by sample (solute) molecule only.

E.gs: UV visible detector, photodiode array detectors, fluorescence detectors and electrochemical detectors.

Bulk Property Detectors

Refractive Index Detectors

They measure the changes in the refractive index of the column effluent passing through the sample cell. Hence, they are also called as *refractive index detectors*. Basically, refractometers are of the following two types.

Deflection Refractometers

These consist of two cells, sample and reference cells which are separated from each other by means of a diagonal glass sheet. Initially, light from a suitable source is allowed to pass through these cells and then via the beam splitter (B_s) is made to fall on two photo cells (PC_1 and PC_2). The refractometer continuously monitors the difference in refractive indices of the mobile phase and the eluent. Due to changes in the refractive index, the beam of light gets deflected by the movement of B_s. The movement of beam splitter is proportional to difference in refractive index which in turn indicates the amount of solute present in column eluent.

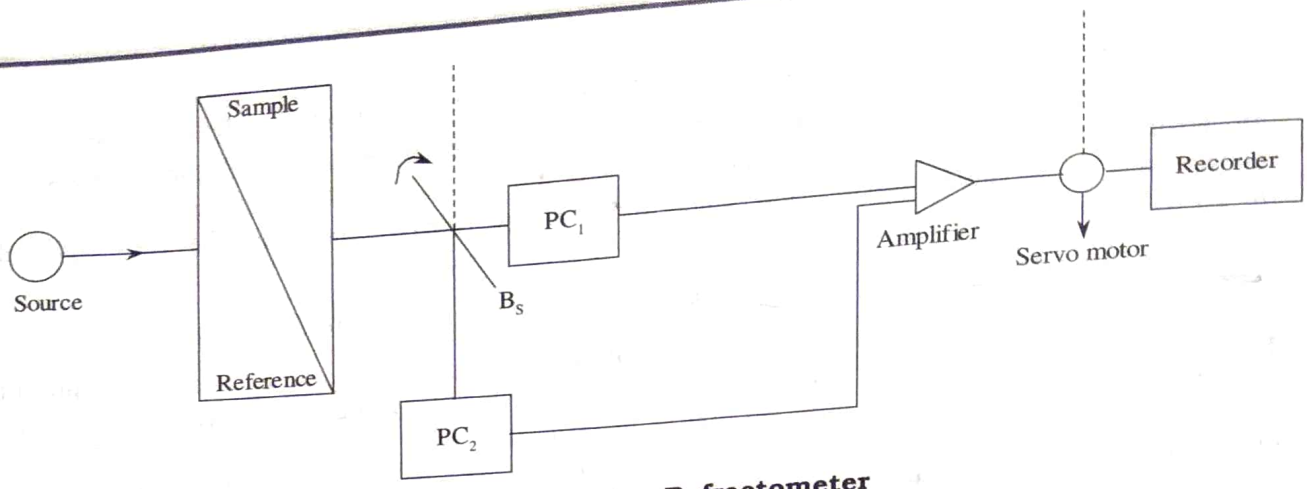

Figure: Deflection Refractometer

Fresnel Refractometers

These refractometers measure the changes in the refractive index of the solvent which cause a change in the reflected and transmitted light at the glass-liquid interface. Similar to deflection refractometer, the light beam from the column and the reference solvent is allowed to pass through the prism and then to the photocell. A change in the amount of transmittance would be encountered when the column eluent contains suitable amounts of solute.

Advantages

1. Refractometers can be used to detect those compounds which do not absorb in the UV region.
2. They are not affected by flow rate and are reliable.
3. There is no requirement of any special groups in structure of molecule (double or triple bond, aromaticity, etc.) for detection.

Demerits

1. They do not possess high sensitivity.
2. Gradient elution cannot be carried out.
3. Demands strict control of temperature during their working.

Solute Property Detectors

UV/Visible Absorption Detectors

They are most widely used detectors in HPLC. They work on the principle of Beer's law i.e., they measure the ability of the sample to absorb light at one or more wavelengths. UV visible detectors are used to detect the solutes that specifically absorb UV/visible radiations.

E.gs: Aromatic compounds and compounds having multiple bonds between carbon, oxygen, nitrogen and sulphur, alkenes, etc.

Two types of UV/visible absorbance detectors are available.

(i) Fixed Wavelength Detectors: They measure the sample absorbance at fixed wavelengths like 245, 250, 280, 334 and 436 nm. Desired wavelength is obtained by allowing the radiations emitted by the mercury vapour lamp to pass through an optical filter. Generally, these detectors are operated with a zinc lamp to produce a wavelength of 214 nm as most of the organic substances absorb light at this wavelength.

(ii) Variable Wavelength Spectrophotometers: They measure the sample absorbance between wavelengths 190-700 nm. They consist of either deuterium or tungsten lamp as the radiation source, a diffraction grating for wavelength selection and a photomultiplier detector for detection.

Photodiode Array Detector

It is a modified form of UV/visible absorbance detector that operates between 190-600 µm. In this, a polychromatic light is made to pass through the flow cell and the emerging radiations obtained are diffracted by a grating and made to fall on an array of photodiodes. Each photodiode receives a different wavelength band which is scanned by a microprocessor and the resulting spectrum is obtained as a three-dimensional plot of response time vs wavelength on a visual display unit.

Advantages

1. High sensitivity.
2. Results are free from interferences since most of the common solvents used do not absorb in the UV/visible range.
3. Not affected by changes in temperature and solvent flow rate.

Fluorescence Detectors

Some inorganic and organic compounds (like polycyclic aromatic hydrocarbons, alkaloids, quinolines) exhibit natural fluorescence and are detected by using fluorescence detectors which measure the fluorescent radiations. Non-fluorescent compounds can be detected by reacting them with appropriate fluorogenic reagents.

In these detectors, radiations emitted from a xenon or deuterium lamp are focussed on the flow cell containing the column eluent through a filter. The fluorescent radiations emitted by the sample are measured at 90° to the incident beam. The desired radiation is picked up by the second filter and detected by using a photomultiplier detector.

Advantages

1. Increased sensitivity and specificity.
2. Independent of changes in flow rates, pressure, temperature etc.

Disadvantages

1. Only fluorescent compounds or those that can be derivatized can be detected.
2. The solvents used should be pure and devoid of any impurities which exhibit fluorescence.

Electrochemical Detectors

In HPLC, it refers to either amperometric detector or coulometric detector. They measure the current associated with the oxidation/reduction of solute at a suitable electrode. The mobile phase used for detection should exhibit conductivity. Inorganic salts or mixtures of water and miscible organic solvents are generally used as conducting mobile phase. Compounds such as aldehydes, ketones, aromatic amines, heterocyclic nitrogen compounds, phenols etc., are detected by electrochemical detectors.

7. Recorders and Integrators

They record the signals emerging from the detector as deviations from the base line. The electrical signals obtained from the detector are amplified (if necessary) and recorded as a function of time with the help of a potentiometric recorder. The responses are obtained in the form of chromatographic peaks from which retention time of the solute molecules is determined. However, the height/area of the peaks which gives a direct measure of the concentration of a component in the mixture cannot be determined.

Integrators are improvised version of recorders. They possess data processing ability and record the individual peaks with their retention time, height, width, peak area, percentage of area etc. Therefore, they help to overcome the disadvantage associated with detectors.

Working of HPLC

The solvents are stored in their respective reservoirs and are delivered into the mixing chamber, where they undergo homogenous mixing to form a required mobile phase. This mobile phase is then delivered into the column under high pressure by means of an appropriate pump. Between the column and the pump is a port through which the sample is injected. The flow rate of the mobile phase (at high pressure) is maintained at 1-2 ml/min. The components of the mixture undergo separation by virtue of their differences in affinity towards the stationary phase. The effluent from the column is then passed through a detector which detects a specific property (UV absorbance, refractive index or fluorescence) which produces electrical signals proportional to the characteristics of the solute molecules. These signals may be amplified and recorded using a potentiometric recorder/integrator and are presented in the form of chromatographs.

2.5 HPLC Techniques

HPLC techniques can be divided into the following types.

1. Based on Modes of Chromatography

There exist two modes of chromatography depending upon the polarity (polar or non-polar) of the stationary and the mobile phases used.

(a) ***Normal Phase HPLC (NP-HPLC)***

It is a type of partition chromatography where stationary phase is polar and the mobile phase is less polar or non-polar. In this mode of chromatography, when the non-polar analyte particles are passed over the polar stationary phases, the analyte particles because of their less affinity towards the stationary phase travel faster than the polar molecules and get eluted from the column. The polar solute particles showing greater affinity towards the polar stationary phase remain in the column for a longer period of time, thus taking more time to get eluted. Each and every analyte elutes from the column at a characteristic time. The effluent is passed through a suitable detector and the chromatographic peaks are recorded. These resulting peaks indicate the identity and quantity of the analyte.

Advantages

1. This technique is simple, accurate and precise.
2. Although the runtime for every injection is more than that of RP-HPLC, NP-HPLC gives out better resolutions and peak symmetry.
3. NP-HPLC can be combined with other suitable chromatographic techniques to develop a comprehensive purification and analysis strategy for compounds which are difficult to analyze.

Disadvantage

Since most of the drug molecules are polar in nature, application of this technique for the separation of such molecules is not considered economical.

(b) ***Reverse Phase-HPLC (RP-HPLC)***

RP-HPLC is the most commonly practised HPLC technique for the analysis of wide range of compounds. In this, the stationary phase used is non-polar while the mobile phase is polar in nature. The term reverse phase is used because unlike in NP-HPLC, the most polar compounds are eluted first while the non-polar compounds are retained for longer durations. Since most of the drug molecules are polar, this technique is widely used in their analysis.

Silica-based columns are the most commonly employed stationary phases in RP-HPLC. The mobile phase may include a mixture of water, aqueous buffers and organic solvents with high dielectric constant like methanol, acetonitrile, tetrahydrofuran, formamide, glycols etc. pH of the mobile phase plays an important role in the retention of an analyte. It can also alter the hydrophobicity of certain analytes. Therefore to control the pH, suitable buffers are added to the mobile phase. Addition of water and organic solvents to the mobile phase results in an increase and decrease in the retention time of polar analytes respectively.

Advantages

1. Water being cheaply available and non-toxic in nature can be used as mobile phase.
2. It can be used for qualitative analysis of drugs in their clinical and preclinical trials.
3. It helps to acquire optimum selectivity and retention with compounds having predominant aliphatic or aromatic character.
4. The technique may be preferred for the separation of analyte molecules depending upon the size and structure of alkyl groups.

Disadvantages

1. Exposure of columns to corrosive acids for longer duration may corrode the metal parts of HPLC instruments.
2. Reverse phase columns when used with aqueous bases result in the destruction of the underlying silica particles.
3. The technique is less sensitive to polar impurities present in the eluent.

Applications

1. This technique can be applied for separation and purification of biochemical compounds, for both analytical and preparative purposes.
2. Used for the analysis of food preservatives, sugars and drugs like analgesics, sedatives, barbiturates, antiepileptics etc.
3. Separation of lipophilic compounds like triglycerides.

2. Based on Principle of Separation

(a) *Adsorption Chromatography*

In this, separation depends upon the relative affinities of the solute molecules towards the stationary phase. The solute molecules having greater affinity towards the stationary phase take longer time to get eluted from the column while those having less affinity elute faster.

(b) *Ion-exchange Chromatography*

The principle involved in separation is the reversible exchange of ions (functional groups) which takes place between ion exchangers present on the solid support and ionic solute molecules. This technique is used to separate the mixtures containing similar charged ions.

A cation exchange resin is used for the separation of cations and an anion exchange resin is used for the separation of anions from the solute mixture.

(c) ***Ion-pair Chromatography***

This technique is used for the separation of mixtures containing wide variety of ionic and ionizable molecules. It utilizes a standard reverse phase column which is converted temporarily into an ion exchange column using ion pairing reagents (E.gs: Pentane/Hexane/Heptane/Octane Sodium Sulphonate, Tetramethyl/Tetraethyl ammonium hydroxide).

(d) ***Gel Permeation/Size Exclusion Chromatography***

The principle involved in separation is the difference in the sizes and shapes of the solute molecules. Different types of gels are used which act as sieves for the separation of molecules of different shapes and sizes under the influence of steric or diffusion effects.

E.gs: Soft gels-Agarose, Dextran, Semirigid gels-Polystyrene.

(e) ***Affinity Chromatography***

In this technique, separation of components from a mixture takes place depending upon the affinity of the solute molecules towards specific stationary phase. It is widely employed in the field of biotechnology, microbiology etc., for the separation of enzymes, nucleic acids etc.

(f) ***Chiral Phase Chromatography***

This technique is employed for the separation of optical isomers using chiral stationary phases. The principle involved in separation differs with the type of stationary phase used and the solute molecules to be separated.

3. Based on Elution Technique

Based on the type of elution technique, there are two types of HPLC techniques.

(a) ***Isocratic Separation***

An elution technique in which the components of mobile phase are maintained constant throughout the process of separation is known as *isocratic elution*. In this, the mobile phase may consist of a single or two or more solvents of similar polarity to elute the sample through the column. E.gs: Chloroform, benzene : pet ether (1 : 1).

During the development process, if the separated constituents of the sample mixture are observed on the column as bands, then the development process may be stopped at that moment and the separated constituents may be extracted by using suitable solvents. Alternatively, the development process may be allowed to continue until the separated constituents elute from the column and reach the detector.

In this type of elution technique, the selectivity does not change even upon changing the length and/or internal diameter of the column. Hence it is preferred only in cases, when the sample mixture contains less than 10 weakly retained components and when the gradient base line negatively affects trace analysis.

Advantages

1. Components of mobile phase remain constant throughout the process. Hence, it becomes easy to handle and does not require continuous monitoring.
2. Any change in column dimensions does not change the elution order (peaks are of same order).

Disadvantages

1. Late eluting peaks are flat and broad.
2. Consumes longer time than gradient elution for complex mixtures.

(b) ***Gradient Separation***

Gradient HPLC also known as solvent programming is a technique employed in HPLC mainly to accelerate the elution of strongly retained components of the analyte from the column and also to resolve the least retained components. During the separation process, the mobile phase composition is continuously changed in a stepwise manner depending upon the interactive character of the stationary phase. This makes the mobile phase steadily stronger as the separation proceeds.

This separation technique is to be started with low concentration of the organic component of mobile phase, the other component being water. The strongly retained components at such concentration of mobile phase either retain strongly on the adsorbent surface of the column or move very slowly. Upon steadily increasing the concentration of the organic component of mobile phase, the competition for adsorption increases. Therefore, the strongly retained components from the column start moving quickly.

The commonly employed organic solvents include methanol, acetonitrile, isopropanol, THF etc. The two liquids i.e., water and organic solvent employed as mobile phase may or may not be miscible with each other but vary considerably in polarity and ionic strengths.

The acceleration of movement of analytes from the column decreases the retention time of slow eluting components. Hence, they give narrower and taller peaks.

Advantages

1. This technique increases the efficiency of columns.
2. Complex sample mixtures with wide retention range can be separated.
3. Samples which contain a variable and/or unknown composition can be screened.
4. Retention of late eluting components is reduced. Hence, they elute faster and give narrower peaks.

Disadvantages

1. Requires continuous monitoring.
2. Maintenance cost is high.
3. Additional mixing of liquids causes a delay in the formation of gradient.
4. Any change in column dimension changes the elution order (peaks are of different order).

4. Based on Scale of Operation

There exist two types of HPLC techniques based on the scale of operation.

(a) ***Analytical HPLC***

This technique as the name suggests, is employed only for the analysis of the sample. Since the quantity of the sample being used is very low, its recovery for re-utilization is not feasible.

(b) ***Preparative HPLC***

In this technique, after the analysis, individual fractions of the pure compounds are recovered using fractional collector and are re-utilized.

2.6 Derivatization Technique in HPLC

Derivatization technique modifies specific functional groups of the reacting compound to a suitable derivative in order to render them analyzable by the desired analytical techniques. This technique is considered as an integral part of a large number of analytical procedures practiced in pharmaceutical, medical and food sciences. It is commonly used in chromatographic analysis for the following reasons.

1. To enhance the thermal stability of compounds by reducing their thermal degradation.
2. To analyze the compounds with insufficient stability or volatility.
3. To prepare volatile derivatives of non-volatile compounds for GC analysis.
4. To prepare soluble derivatives of insoluble compounds for HPLC analysis.
5. To prepare the fluorescent derivatives of compounds in order to make them specifically detectable.
6. To decrease the polarity of compounds.
7. To enhance separation.
8. To reduce tailing, poor peak resolutions and/or unsymmetrical peaks.
9. To improve the chromatographic detector response by incorporating functional groups in the derivatives that are capable of eliciting higher detector signals.

Derivatization technique in HPLC can be carried out in one of the following modes.

1. Pre-column Mode

This mode of derivatization is performed before the analytical separation is attained. The sample is derivatized manually or automatically and the stable derivatives are then injected into the HPLC column. Hence, the separation of components of the sample occurs after derivatization.

Table: Examples of Pre-column Derivatization Reagents

Reagent	Applications
Benzylamine	For derivatization of plasma and 5-hydroxyindoles
Phthaldialdehyde	For derivatization of ethylamine, histamine, tryptamine etc
9-fluoromethyl chloroformate	For derivatization of aminoglycosides and amphetamines

2. Post-column Mode

Post-column derivatization is commonly employed for the separation of antibiotics, amino acids, peptides, proteins, toxins, carbamates as well as for those compounds that show zero or very little UV absorbance.

This mode of derivatization is performed after the analytical separation of compounds. The HPLC instrument is modified by the addition of a secondary pump placed in between the column and the detector. The sample is first injected into the column in usual manner and the derivatization reagent is automatically introduced into the separated molecules via the secondary pump. These pumps are advantageous as they can dispense volumes of derivatization reagents as low as 0.25 µl with the minimal flow rate of 150 µl/min. The derivatization reagent must be capable of reacting quickly and should be compatible with the mobile phase. Moreover, it should not cause any rearrangements or structural alterations in the formed derivatives.

Table: Examples of Post-column Derivatization Reagents

	Reagent	Examples	Applications
1.	Non-fluorescent reagent	4-fluoro-3-nitrobenzotrifluoride	For derivatization of polyamines like spermidine, spermine and putrescine
2.	Fluorescent reagent	4-hydrazine-7-nitro-1, 2,3-benzoxadiazole hydrazine	For derivatization of primary and secondary amines
3.	Chiral derivatization reagent	2,3,4-tri-*o*-acetyl-α-D-arabinopyranosyl isothiocyanate	For derivatization of biogenic chiral amines

2.7 Applications

1. It is used in inorganic chemistry for separating anions and cations.
2. It is used in forensic science for the separation of phenylalkyl amines (morphine and its metabolites) from blood plasma and for the detection of poisons or intoxicants such as alcohol, carbon monoxide, cholinesterase inhibitors, heavy metals, hypnotics etc.
3. It is used in environmental studies for analyzing the pesticide content in drinking water.
4. It is used in food analysis for separating water soluble and fat soluble vitamins from variety of food products, fortified food and animal feed.
5. It is used for determining the quantities of antioxidants and preservatives present in the food.
6. It is used in cosmetic industry for the assay and quality control of various cosmetics like lipsticks, creams, ointments etc.
7. It is used for separating various components of plant products which bear structural resemblance.

   Example: Analysis of cinchona, digitalis, ergot extracts and liquorice.
8. It is used in agrichemical industry for separation of herbicides.
9. It is used in the separation and analysis of amino acids, carbohydrates, proteins, lipids and steroidal hormones.
10. It is used for separating coal and oil products from their crude sources.
11. It is used for the separation and identification of psychotropic drugs such as antidepressants, benzodiazepines, butyrophenones, neuroleptics, phenothiazines etc.
12. It can be used for determining the stability of various pharmaceuticals by analyzing the degradation products of drugs.
13. It can be used in bioassays of compounds like chloramphenicol, cotrimoxazole, penicillins, peptide hormones and sulphonamides.
14. It is used for controlling microbiological processes used in the production of number of antibiotics such as chloramphenicol, tetracyclines, penicillins and streptomycin.
15. It is used for monitoring the course of organic synthesis and also for isolating products in the reaction.
16. Used for the separation of required enantiomer from a mixture of stereoisomers.

17. It gives an idea about the biopharmaceutical properties of a dosage form and the pharmacokinetics of the drugs. Therefore, it is used in dosage form design.

18. It is used as an analytical method for numerous natural and synthetic drugs. It is used in different levels of pharmacy and pharmacology, such as for control of raw materials, isolation of pharmaceuticals and in production, development and product control.

19. It is used in nucleic acid research for numerous purposes like,

    (a) For studying the regulatory effects of cyclic nucleotides

    (b) For determining the composition of hydrolysates of nucleic acids

    (c) For studying the diseased processes

    (d) For metabolic profiling of normal and diseased subjects

    (e) For separation and purification of nucleic acids.

Table: Comparison between GC and HPLC

	Gas chromatography (GC)	High performance liquid chromatography (HPLC)
1.	In this technique, a stream of carrier gas (mobile phase) is passed over a fixed stationary phase placed inside the column.	In this technique, a liquid mobile phase is pumped at a high pressure through a suitably modified column that acts as the stationary phase.
2.	The principle involved in GLC is partition while in GSC, it is adsorption.	The fundamental principle of separation in HPLC is adsorption.
3.	Instruments used are simple, inexpensive and rugged.	HPLC columns are expensive and the maintenance cost of the equipment is high.
4.	Gaseous and volatile compounds can be easily separated and analyzed.	Gaseous and volatile compounds cannot be separated and analyzed.
5.	Since the technique is performed at elevated temperatures, only thermostable compounds can be analyzed.	Thermolabile compounds can be easily separated and analyzed.
6.	Preparation of mobile phase is easy.	Preparation of sample and solvent is a time consuming process.
7.	Mobile phase cannot be recovered.	Mobile phase can be recovered.
8.	Sample needs to be heated at elevated temperatures.	No sample pretreatment is required when aqueous or non-aqueous samples are to be analyzed.
9.	It requires temperature programming.	Temperature programming is not essential.
10.	Instrumentation includes gas supply unit, sampling unit, column unit, detector and recorder.	Instrumentation includes mobile phase reservoir and solvent pretreatment systems, solvent delivery pumps, precolumn/guard column, sample injection systems, analytical columns, detector and recorder.

	Gas chromatography (GC)	High performance liquid chromatography (HPLC)
11.	Precolumn is not required.	A precolumn that increases the efficiency of chromatographic separation is essential.
12.	Sampling unit, column and detector are to be supplied with ovens to maintain higher temperatures.	Only the column unit needs an oven.
13.	Glass columns are most commonly used because they can withstand higher temperatures.	Stainless steel columns are commonly used because they can withstand higher pressures.
14.	Length of the column may vary from a few centimeters to several hundred metres.	Length of the column may vary from 25-100 cm.
15.	Detectors used are thermal conductivity detector, electron capture detector, flame ionization detector, nitrogen phosphorous detector, flame photometric detector etc.	Detectors used are visible detectors, refractive index detectors, conductivity detectors, UV visible detector, fluorescence detector, electrochemical detector etc.